IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit	: 1635
Examiner	: Louis V. Wollenberger
Applicant	: Nariyoshi Shinomiya et al.
Appln. No.	: 10/599,327
Filing Date	: March 12, 2007
Confirmation No.	: 7013
For	: *c-met* siRNA ADENOVIRUS VECTORS INHIBIT CANCER CELL GROWTH, INVASION AND TUMORIGENICITY

DECLARATION UNDER 37 C.F.R. § 1.131

We, the undersigned, do hereby declare as follows:

1. We are the co-inventors of the claims of the above-identified patent application.

2. The invention as defined in claims 1-12, 14-17, and 38 was conceived of and actually reduced to practice prior to January 6, 2003. The invention as defined in claims 13, 18-20, and 48-50 was conceived of and actually reduced to practice prior to July 7, 2003.

3. Evidence of our conception and reduction to practice of the invention as defined in claims 1-20, 38, and 48-50 is provided in the form of experimental data from the laboratory notebook of Nariyoshi Shinomiya, one of the named inventors (attached hereto as Exhibit A1-A10). More specifically, these laboratory notebooks show our development of an RNAi molecule directed to c-met:

a) in the cancer cell lines DU-145, SK-LMS-1, DA3, and M114 (Exhibit A1);

b) using siRNA expression vector pSilencer 1.0-U6 for human c-met (Exhibit A2 and A4);

c) targeting human c-met sequence 221, the target of SEQ ID No. 15 (Exhibit A3);

d) using pShuttle vector (Exhibit A5);

e) using an Ad5 viral vector (Exhibit A6 and A9);

f) using a stable transformant (Exhibit A7 and A8); and

g) in DBTRG glioblastoma cells (Exhibit A10).

4. The documents attached as Exhibit A1-A10 were prepared contemporaneously with our conception and reduction to practice.

5. The acts referred to in the preceding paragraphs occurred in the United States.

6. The undersigned hereby declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Sections 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

September 30, 2009
Date

George F. Vande Woude

September 26, 2009
Date

N. Shinomiya
Nariyoshi Shinomiya

< Cell culture from the stock >

① DU-145 ——— RPMI 1640 + 5% FBS
(−160°C stock = 1/11/02) (+ 1% pen/strep)

② SK-LMS-1 ——— DMEM + 10% FBS
(passage 2) Atcc (#11965-092) (+ 1% pen/strep)

③ DA3 ——— DMEM + 10% ~~Calf Serum~~ Fetal Bovine Serum
(−160°c stock = 3/23/01) (+ 1% pen/strep)

④ M114 ——— DMEM + 10% ~~Calf~~ Serum
(−160°C stock = 10-26-01) or Fetal Bovine Serum
(+ 1% pen/strep)

Culture media & Cell preservation media

* 5% FBS-RPMI 1640 (Cat No: 11875-093
Lot No: 1104467
FBS 15 ml
P/S 3 ml
RPMI 282 ml

* 10% FBS-DMEM (Cat No: 11965-092
Lot No: 1143007
FBS 30 ml
P/S 3 ml
DMEM 267 ml

* Cell preservation media
DMSO 2 ml
FBS 2 ml
RPMI 23 ml
or
DMSO 7 ml
FBS 20 ml
DMEM 73 ml

<Transformation of pSilencer™ 1.0-U6 vector
into TOP10 competent E. coli>

TOP10 → from freezer

↓

50 µl → eppendorf tube on ice

↓

pSilencer 1.0-U6 0.7 µl (= 150 ng)

↓

25 min on ice

↓

30 sec at 42°C

pSilencer 1.0-U6

↓

add 250 µl SOC

↓

Shaking incubation (37°C, 250 rpm)

↓

plate on amp LB plate (50 µl, 100 µl)

<continued>

100 µl → colonies are too many (can not be picked up)

50 µl → ~ 2000 colonies

↓

5 colonies are picked up

↓

duplicated plate ① → plasmid purification

② → stock of cells

LB-amp

↓

37°C

< Transformation mouse siRNA — part 4 >

① LB amp Agar

		pick up
#57	8 colonies	(1, 2, 3)
#60	1 colony	(1)
#110	1 "	(1)
#120	0 "	X
#178	5 colonies	(1, 2, 3)

→ LB amp medium and shaking incubation (7 = 0.5 ~)

* Transformation efficiency was not very good!

Since human siRNA oligo transformation obtained a good result, maybe there is a problem in the core of siRNA oligo.

↓

Try again by using higher conc of siRNA oligos

↳ #60 #110 #120 only

② Ligation again

miRNA pre BbsI/XbaI digest & gel purified	0.5 μl
X μM oligo	2 μl
5 x Ligase buffer	2 μl
water	5 μl
T4 DNA Ligase	0.5 μl
Total	10 μl

→ Incubate at RT for 30 min

All EDTA inactivated

→ transformation in the same way as previously — LB amp

(~ 7.5hr incubation 37°C, 250 rpm)

4°C 25 hr

→ plasmid extraction using QIAGEN Miniprep kit

↓

Analysis by electrophoresis → ⑧ page 22b

<Confirmation of the extracted plasmids>

① Digestion of plasmids with restriction enzymes

#57, #178 → XbaI (Buffer 2)

#16-1,2,3,4 #62-1,2,3,4 #76-1,2,3,4 #178-1,2,3,4 → HindIII (Buffer 2)

* 1μl each plasmid was digested at 37°C for 1 hr

② Electrophoresis (1% agarose gel, GENE Mate 150V for 1.5hr)

* gel stain → 150ml water + 30μl EtBr (10 mg/ml)

1 — λ HindIII
2 — mU6 (circular)
3 — #57
4 — #57/XbaI
5 — #178
6 — #178/XbaI (#178-2)
7 — λ HindIII
8 — pSilencer (circular)
9 — pSilencer/HindIII (2kbp)
10 — #16-1
11 — " 2
12 — " 3
13 — " 4
14 — #62-1
15 — " 2
16 — " 3
17 — " 4
18 — #76-1
19 — " 2
20 — " 3
21 — " 4
22 — #221-1
23 — " 2
24 — " 3
25 — " 4

HindIII digest

(A) Mouse siRNA

XbaI digest → 3.4kb is OK

(mU6 ~4143 nt)
mU6 BbsI/XbaI ≒ 3.4kb
mU6 insert ≒ 3.4kb

(B) human siRNA

after HindIII digestion still circular is OK (≒3.3kb)

○ → to be sequenced!

③ Sequencing

- #178 (#128-0) → mouse siRNA for c-Met (M13 R2 primer)
- #16 (#16-3)
- #62 (#62-2)
- #76 (#76-1)
- #221 (#221-2)

#16, #62, #76, #221 → human siRNA for c-Met (T3 primer (A))

Template plasmid [illegible] μl (~2 μg)
Primer 1 μl (3.2 pmol)

↳ Sequence [illegible]

* Primers

M13 R2 = 6[illegible] pmol → dissolved in [illegible] μl of water (= 300 pmol/μl)

T3 (A) = [illegible] pmol → dissolved in [illegible] μl of water (= 300 pmol/μl)

→ store at -20°C

↓

Then 10 fold dilution with water
primer : water = 10 μl : 90 μl → use 1 μl each for sequencing

↗ Stock (250 pmol/μl) -20°C

siRNA hairpin template sequences for human c-met RNAi

A. Criteria of Sequence Selection

1. 21mer that start with AA
2. GC content between 45-55%
3. No more than three consecutive T or G nucleotides can be present anywhere in the hairpin template sequences
4. The targeted region is selected from a given cDNA sequence beginning 50 to100 nt downstream of the start codon. (5' or 3' UTRs and regions nearby the start codon are avoided, as these may be richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNP or RISC endonuclease complex.)
5. Blasted selected sequences against Genbank
6. Nucleotide cross-match (no more than 17, much less would be better)
7. Start with AAG (for cloning into pSilencer 1.0-U6 vector system; RNA transcription begin with G, but according to the company's information a +1 G is not required.)
8. A pair of template sequences is as follows:

Sense Loop Antisense
Template 1: 5'-N(19)-TTCAAGAGA-N(19)-TTTTTT-3' (53mer)
Template 2: 3'-CCGG-N(19)-AAGTTCTCT-N(19)-AAAAAATTAA-5' (61mer)
ApaI EcoRI

Target sequence (16): AAGACCTTCAGAAGGTTGCTG
Blast search (in new window)
Position in gene sequence: 415
GC content: 47.6%
siRNA Sense strand: GACCTTCAGAAGGTTGCTGTT
siRNA Antisense strand: CAGCAACCTTCTGAAGGTCTT
#16-1: 5'-GACCTTCAGAAGGTTGCTG-TTCAAGAGA-CAGCAACCTTCTGAAGGTC-TTTTTT-3'
#16-2: 3'-CCGG-CTGGAAGTCTTCCAACGAC-AAGTTCTCT-GTCGTTGGAAGACTTCCAG-AAAAAATTAA-5'
#16-2: 5'-AATTAAAAAA-GACCTTCAGAAGGTTGCTG-TCTCTTGAA-CAGCAACCTTCTGAAGGTC-GGCC-3'

Target sequence (62): AAGCCAGATTCTGCCGAACCA
Blast search (in new window)
Position in gene sequence: 1236
GC content: 52.4%
siRNA Sense strand: GCCAGATTCTGCCGAACCATT
siRNA Antisense strand: TGGTTCGGCAGAATCTGGCTT
#62-1: 5'-GCCAGATTCTGCCGAACCA-TTCAAGAGA-TGGTTCGGCAGAATCTGGC-TTTTTT-3'
#62-2: 3'-CCGG-CGGTCTAAGACGGCTTGGT-AAGTTCTCT-ACCAAGCCGTCTTAGACCG-AAAAAATTAA-5'
#62-2: 5'-AATTAAAAAA-GCCAGATTCTGCCGAACCA-TCTCTTGAA-TGGTTCGGCAGAATCTGGC- GGCC-3'

Target sequence 76: AAGCGCGCCGTGATGAATATC
Blast search (in new window)
Position in gene sequence: 1417
GC content: 52.4%
siRNA Sense strand: GCGCGCCGTGATGAATATCTT
siRNA Antisense strand: GATATTCATCACGGCGCGCTT
#76-1: 5'-GCGCGCCGTGATGAATATC-TTCAAGAGA-GATATTCATCACGGCGCGC-TTTTTT-3'
#76-2: 3'-CCGG-CGCGCGGCACTACTTATAG-AAGTTCTCT-CTATAAGTAGTGCCGCGCG-AAAAAATTAA-5'
#76-2: 5'-AATTAAAAAA-GCGCGCCGTGATGAATATC-TCTCTTGAA-GATATTCATCACGGCGCGC- GGCC-3'

Target sequence (221): AAGTGCAGTATCCTCTGACAG
Blast search (in new window)
Position in gene sequence: 3310
GC content: 47.6%
siRNA Sense strand: GTGCAGTATCCTCTGACAGTT
siRNA Antisense strand: CTGTCAGAGGATACTGCACTT
#221-1: 5'-GTGCAGTATCCTCTGACAG-TTCAAGAGA-CTGTCAGAGGATACTGCAC-TTTTTT-3'
#221-2: 3'-CCGG-CACGTCATAGGAGACTGTC-AAGTTCTCT-GACAGTCTCCTATGACGTG-AAAAAATTAA-5'
#221-2: 5'-AATTAAAAAA-GTGCAGTATCCTCTGACAG-TCTCTTGAA-CTGTCAGAGGATACTGCAC- GGCC-3'

< Sequence conformation of siRNA plasmid >

Expression plasmids for c-met RNAi (final clones)

1. Mouse siRNA (host plasmid = mU6pro)

Target sequence No.	Position in gene sequence	Ligation of synthesized oligos & transformation	Sequence confirmation of the inserted oligos	Final clone #s
#57	950	finished	confirmed	#57-1, #57-2, #57-3
#60	988	finished	confirmed	#60-1, #60-4
#110	1839	finished	confirmed	#110-1, #110-2, #110-3
#120	1977	Very low transformation efficiency	-	-
#178	2762	finished	confirmed	#178-0, #178-1, #178-2

2. Human siRNA (host plasmid = pSilencer)

Target sequence No.	Position in gene sequence	Ligation of synthesized oligos & transformation	Sequence confirmation of the inserted oligos	Plasmid amplification for transfection
#16	415	finished	confirmed	#16-3
#62	1236	finished	confirmed	#62-2
#76	1417	finished	Wrong sequence	-
#221	3310	finished	confirmed	#221-6

#red, #blue : plasmid clones were amplified and used for transfection
Control plasmid: mU6pro = mU6#1, pSilencer = pSil#1

< plasmid amplification — #110-1 #221-6 #mU6#1 pSil#1 >

2ml → shaking incubation

LBamp

#110-1
#221-6
→ from LB plate

#mU6#1
pSil#1
→ from -80°C stock

160ml overnight shaking incubation !!

< Large-Scale Plasmid Amplification
— Making cMet siRNA expression plasmids >

(1) Yesterday — separation cultures from -80°C stocks
↓
(2) Today — pick up one colony & shaking cultures in 2 mL LB-Amp (100 μg/mL) [Green ...]

Ligation reaction (brief protocol)

a. Stick-ends (Cohesive Ends) Ligation

1. To an eppendorf tube add the following:

5x ligase reaction buffer	4 μL
vector DNA	3-30 fmol (2.5-25 ng)
insert DNA	9-90 fmol (7.5-75 ng)
(total DNA	0.01-0.1 μg)
autoclaved distilled water	to 19 μL
T4 DNA Ligase	1 unit (in 1 μL)
Final volume	20 μL

2. Mix gently. Centrifuge to bring the contents to the bottom of the tube.
3. Incubate at 23°C to 26°C for at least 5 min (30 min would be better).
4. Add 1 μL of 0.5 M EDTA to inactivate the enzyme.
5. Store the reaction at 4°C.
6. Dilute an aliquot of the ligation reaction five-fold in autoclaved distilled water and use it to transform competent cells.

b. Blunt ends Ligation

1. To an eppendorf tube add the following:

5x ligase reaction buffer	4 μL
vector DNA	15-60 fmol (25-250 ng)
insert DNA	45-180 fmol (75-750 ng)
(total DNA	0.1-1.0 μg)
autoclaved distilled water	to 19 μL
T4 DNA Ligase	1 unit (in 1 μL)
Final volume	20 μL

2. Mix gently. Centrifuge to bring the contents to the bottom of the tube.
3. Incubate at 14°C for 16-24 hr.
4. Add 1 μL of 0.5 M EDTA to inactivate the enzyme.
5. Store the reaction at 4°C.
6. Dilute an aliquot of the ligation reaction five-fold in autoclaved distilled water and use it to transform competent cells.

Design (restriction enzyme digestion & ligation)

a. Murine c-met siRNA

pShuttle (vector): XbaI/HindIII double digestion --> gel purification
mU6pro (insert): HindIII/XbaI double digestion --> gel purification

XbaI = REact 2, 37°C, 1 hr
HindIII = REact 2, 37°C, 1 hr

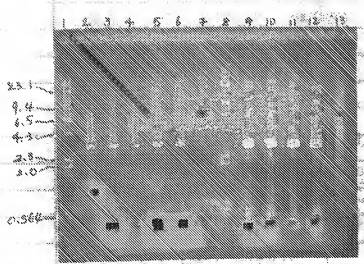
*stick ends ligation (direction of the insert is reverse)

b. Human c-met siRNA

pShuttle (vector): KpnI digestion --> phenol extraction/ethanol precipitation
EcoRV digestion --> gel purification
pSilencer (insert): KpnI/SmaI double digestion (30°C then 37°C) --> gel purification

KpnI = REact 4, 37°C, 1 hr
SmaI = Gene Choice buffer 4 (=REact 4), 30°C, 1 hr
EcoRV = Gene Choice buffer 2 (=REact 2), 37°C, 1 hr

*blunt ends ligation (the same direction)



1 — λ HindIII
2 — mU6 — ~1.2 kbp
3 — 57-1
4 — 60-1 — HindIII/XbaI double digest
5 — 110-1 — ~400bp
6 — 178-1
7 — pShuttle — 6.6 kbp
8 — λ HindIII
9 — pSil
10 — 16-3 — KpnI/SmaI double digest (~400bp)
11 — 62-2
12 — 221-6
13 — pShuttle – KpnI/EcoRV digest (~6.6kbp)

desired bands were recognized!!

gel purification

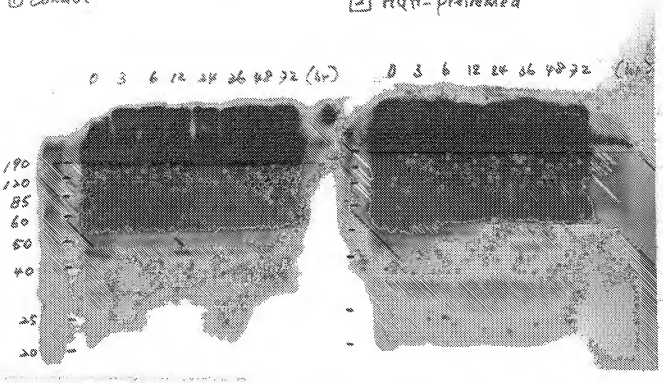
elute with H_2O (final vol = 30μl) (store -20°C until use)

Tomorrow Ligation Reaction

< Western blot ~ Results >

① Control

② HGFI-pretreated



* 50 ~ 190 KDa ⇒ phosphorylation bands are too strong

↓

[Next time load 50μg protein
[Separate with 8% gel

< Adenovirus purification using Virapur >

	OD 260	280	260/280	titre (cpu/ml)
pAd ②	0.161	0.135	1.195	1.8×10^{12}
pAd ③	0.186	0.125	1.495	2.0×10^{12}

pAd ④
pAd ⑤
] → harvest tomorrow

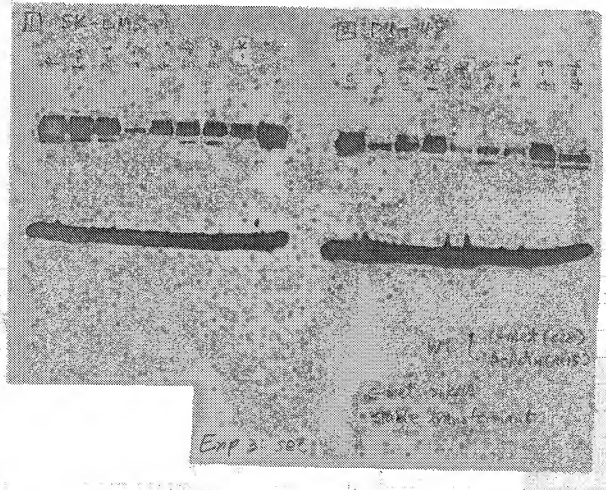
pAd ② ③
300μl each × 9
+ 1 each
↳ -80°C

< Western blot ~ siRNA transfectant
SK-LMS-1 & DU-145 >

primary Ab
(c-Met (C28) 1:2000
(β-Actin (AC15) 1:5000

secondary Ab
(α-rabbit 1:1000
(α-Mouse 1:5000

@ protein
20μg was Loaded in each lane
(both SK and DU)



C-Met Downregulation is also observed in DU-145 cells

< Histology of the remnant livers >

Serial number	Sample name	Serial number	Sample name
GVW-4001	C 12h-1	GVW-4016	H 12h-1
4002	C 12h-2	4017	H 12h-2
4003	C 12h-3	4018	H 12h-3
4004	C 24h-1	4019	H 24h-1
4005	C 24h-2	4020	H 24h-2
4006	C 24h-3	4021	H 24h-3
4007	C 36h-1	4022	H 36h-1
4008	C 36h-2	4023	H 36h-2
4009	C 36h-3	4024	H 36h-3
4010	C 48h-1	4025	H 48h-1
4011	C 48h-2	4026	H 48h-2
4012	C 48h-3	4027	H 48h-3
4013	C 72h-1	4028	H 72h-1
4014	C 72h-2	4029	H 72h-2
4015	C 72h-3	4030	H 72h-3

Difference in the mitotic indices ?

4001 ~ normal, hard to find mitotic cells
4002 ~ small vacuoles ⊕, "
4003 ~ bleeding inside the liver, hyaline-like changes
4004 ~ many vacuoles ⊕⊕
4005 ~ small vacuoles ⊕ (around the vein. novacuoles)
4006 ~ "
4007 ~ vacuoles ⊕
4008 ~ small vacuoles ⊕
4009 ~ small vacuoles ⊕, normal
4010 ~ small vacuoles ⊕/ mitotic cells occasionally
4011 ~ vacuoles ⊕/ mitotic cells, occasionally
4012 ~ " / "
4013 ~ mitotic cells 1~3/HPF
4014 ~ "
4015 ~ "

4016 ~ many bleeding sites
4017 ~ small vacuoles ⊕
4018 ~ " ⊕
4019 ~ vacuoles ⊕⊕
4020 ~ " ⊕⊕
4021 ~ " ⊕
4022 ~ vacuoles ⊕ depends on the portion / mitosis occasionally
4023 ~ vacuoles ⊕, Mitosis occasionally
4024 ~ vacuoles ⊕ hyaline necrosis
4025 ~ vacuoles ⊕ inflammation Mitosis 2~3/HPF
4026 ~ mitosis 2~3 /HPF
4027 ~ "
4028 ~ Mitosis 2~3/HPF
4029 ~ "
4030 ~ "

EXHIBIT A8
Page 1 of 2

< Western blot ~ siRNA stable transformant >
Expression of EGFR

[1] SK-LMS-1

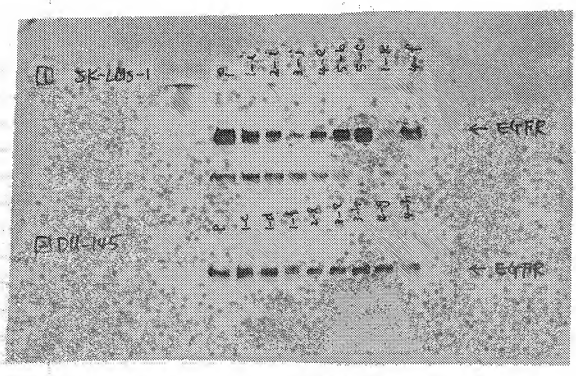
X, M, Ⓟ, 1-i, 2-i, 3-j, 4-e, 5-b, 5-c, 1-k, 4-f, X ← 20μg protein each loaded

[2] DU-145

X, M, Ⓟ, 1-c, 1-d, 1-f, 2-l, 3-e, 3-h, 4-g, 4-h, X

1st Ab : α-EGFR 1:1000
2nd Ab : α-Rabbit 1:2000

Results ~



① { in SK-LMS-1 → expression of EGFR varies & also correlates well with proliferative activity
in DU-145 → no remarkable changes in the EGFR levels

< Large scale virus production ~ pad ① >

T175 × 5 flasks

↓

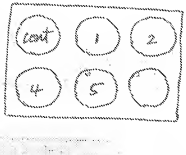
Virapur kit

OD260	OD280	260/280 = 1.554
0.087	0.056	

* cpu/ml = 0.087 × 50 × 1.1 × 10^{12} ≒ 4.8 × 10^{12} (cpu/ml)

500μl each × 8 tubes + 1 additional tube → −80°C stored

< Cell scattering activity ~ BAB cells >



10ng/ml HGF was added to each well
(9:30 a.m. ~ ≒ 0^4)

< Large scale virus production ~ pAd ⑤ >

T175 × 5 flasks ~ almost all cells detached from the bottom and floated as single cells.

↓

Virapur kit

OD 260	OD280	260/280 ratio
0.041	0.029	1.394

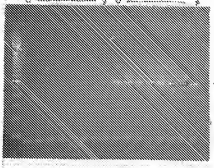
particle number = 0.041 × 50 × 1.1 × 10^{12} ≒ 2.3 × 10^{12} (cpu/ml)

500μl each × 8 tubes + 1 additional tube (>1000μl) → −80°C stode

EXHIBIT A9

< RT-PCR results >

scid cont. scid HGF-Tg

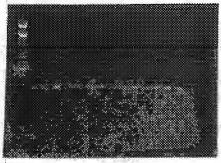


← hHGF (539bp)

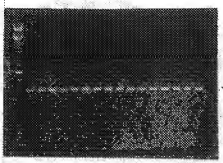
* after 1/3 gH

0	1	2	3	4	5	6	7
0h	8h	6h	12h	24h	36h	48h	72h

↑ no hHGF expression in scid control mice.



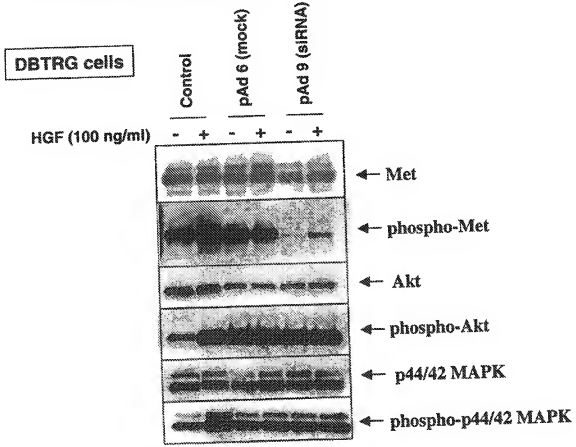
← mHGF (365bp) → no remarkable changes in terms of time course



← m β-actin (376 bp)

< DBTRG cells siRNA adv. infect ⇒ HGF stimulation >

siRNA suppresses Met phosphorylation



- Met is down regulated in pAd⑨ infected cells
- Met phosphorylation is also significantly suppressed in pAd⑨ infected cells
- But regarding to the AKT-phosphorylation & p44/42 MAPK phosphorylation strong phosphorylation bands were observed from the beginning (before HGF stimulation)